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Proto-oncogene c-erbB-2 is overexpressed in 25-30% human breast cancers. Recently, *in vitro* studies have shown that transcription factor AP-2 proteins can bind to a critical region in the c-erbB-2 promoter and activates the promoter. We have therefore begun to determine the role of AP-2 both in normal mammary gland development and in breast cancer. Analysis of human breast cancer biopsies reveals that there is a significant increase in AP-2 protein expression in the progression from benign breast epithelium. Moreover, the presence of various AP-2 family members correlates with the expression of important signal transduction molecules including c-erbB-2, ER and IGF-IR. To test the role of AP-2α in the development of the mammary gland, We have generated transgenic mice with overexpression of either the wild-type or the dominant-negative forms of AP-2α in the mammary gland. Analysis of these mice reveals that AP-2 exerts a profound influence over the growth and morphogenesis of the mammary gland. Overexpression of AP-2α impairs side-branching of virgin mammary gland and inhibits lobuloalveolar development during pregnancy and lactation. This animal model provides a valuable tool to study the role of AP-2α in proliferation and differentiation during the development of the mammary gland.

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Jian Zhang 9-27-99

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Introduction

Breast cancer is one of the most common and important diseases affecting women in North America and Western Europe (1, 3). Despite advances in treatment, only modest increases in survival have been achieved. Examination of the molecular changes in human breast cancer has revealed that one of the most common alterations is the overexpression of the normal c-erbB-2 gene. The normal c-erbB-2 gene (also known as *neu* or HER2) encodes a 185 kDa tyrosine kinase transmembrane receptor which shows extensive structural similarity with the epidermal growth factor receptor (17, 18). The human c-erbB-2 gene, which is normally expressed at low levels in a variety of adult epithelial cells, is overexpressed in 25-30% of carcinomas of the breast (2). Moreover, high levels of c-erbB-2 expression have been shown to correlate with poor prognosis and to predict a worse response to therapy (2, 3).

The potential of the c-erbB-2 gene to cause tumors has been assessed in both cultured cells (4, 5) and transgenic mice (6, 7). In tissue culture it has been shown that the normal human c-erbB-2 cDNA was able to induce transformation of NIH3T3 cells when fused to strong viral transcriptional control elements (4, 5). Furthermore, inappropriate overexpression of the c-erbB-2 gene in mouse mammary tissue can induce mammary carcinoma (6, 7). Finally, a recombinant humanized c-erbB-2 antibody, Herceptin, has been shown to enhance the anti-tumor efficiency of chemotherapy (32-34). Taken together, these data strongly support a role for c-erbB-2 in human disease.

One of the fundamental questions arising from these studies is what is the mechanism of c-erbB-2 overexpression in human mammary carcinomas? In general the increase in c-erbB-2 mRNA and protein is associated with amplification of the gene (2), although it can also occur from a single copy gene (8, 9,16). Indeed, studies in cell lines derived from human mammary tumors have shown that there is a 6-8 fold increase of c-erbB-2 mRNA per template copy in overexpressing cells, whether or not the gene is amplified (8, 9). A critical region of the human c-erbB-2 promoter that is responsible for the activation in overexpressing mammary cancer cells has recently been identified (11). Transfection and DNaseI footprinting assays have led to the identification of a transcription factor, originally termed OB2-1, present in c-erbB-2 overexpressing cells, that binds this critical region and activates the c-erbB-2 promoter (10). More recent studies involving our laboratory have shown that OB2-1 is antigenetically and functionally indistinguishable from the developmentally regulated transcription factor AP-2 (11-14).

Further examination of the OB2-1 complex indicated that it contained other AP-2 related proteins. In particular, peptide sequence derived from OB2-1 demonstrated that it is actually a mixture of three proteins belonging to the AP-2 gene family (19). In addition to the original AP-2 gene, two other novel AP-2 genes present in the OB2-1 complex have been isolated. A comparison of the predicted peptide sequence reveals that these new genes, AP-2 β and AP-2 γ, are highly related to the original AP-2 protein, now termed AP-2 α. The three proteins all recognize the same DNA sequence and can

form either homo- or hetero- dimers with themselves or with each other (19). Furthermore, the three AP-2 proteins are all capable of activating expression of reporter genes driven by the human c-erbB-2 promoter (19). Taken together, these data indicate that the c-erbB-2 gene can act as a target for transactivation by AP-2 proteins.

We and others have recently established the critical importance of AP-2α as a developmental regulator in murine embryogenesis using knockout mouse technology. The AP-2α-null mice have severe defects in many organs including the head, brain, peripheral nerves, limbs, and the ventral body wall where the mammary gland normally resides (20, 21). In contrast, little is known about the role of AP-2α in adult mouse development. Because AP-2α is a fundamental gene regulator and is associated with breast cancer, we have begun to determine the role of AP-2α both in normal development of the mammary gland and in breast cancer.

Since July 1, 1998, I have made significant progress in pursuit of my specific aims. As outlined in more detail below. This first necessitated breeding transgenic mouse lines carrying either the wild-type transgene or the dominant-negative forms of AP-2α to a suitable colony size. Subsequently, I have determined the extent of transgene protein expression in the transgenic lines. I have also analyzed the phenotype of transgenic mouse lines, and I have shown that overexpression of wild-type AP-2α inhibits the differentiation of the mammary epithelium.

Materials and Methods

Tissue Collection and Histological Analysis.

Mouse mammary gland tissue samples were obtained by removing the fourth inguinal gland from mice at various stages of postnatal development, i.e. from virgin, pregnant, lactating and regressing mice. The tissue specimens were fixed with 10% natural buffered formalin or 4% paraformaldhyde, dehydrated in a graded series of ethanols and xylenes, then embedded in paraffin wax. 6 μm sections were cut and mounted on poly-L-lysine coated slides. These sections were stained with hematoxylin and eosin or used for immunohistochemical analysis or *in situ* hybridization. Whole-mount analysis of mouse mammary tissue was performed as previously described (23).

Antibody and Immunohisochemistry.

The analysis of transgene expression was performed with the polyclonal antibody SC-807 (Santa Cruz) which recognizes the FLAG epitope tag. Briefly, sections were incubated with an 1:150 dilution of SC-807 overnight at 4°C and staining was developed using an avidin-biotin peroxidase technique employing the commercially available Vectastain ABC kit (Vector Laboratories).

In situ Hybridization Analysis.

Plasmid pBl-WAP, containing a 295-bp fragment of whey acidic protein cDNA, and plasmid pBl-β-cas, containing a 170-bp fragment of β-casein cDNA, are gifts from Dr. Frank Jones (Yale Medical School). pBl-WAP was linearized with BamH I and a single-stranded antisense radio-labeled probe was synthesized using T7 RNA polymerase in an *in vitro* transcription reaction in the presence of ^{35}S UTP. pBl-β-cas was linearized with Hind III and transcribed with T3 RNA polymerase to generate an equivalent probe for this gene. The *in situ* hybridizations were performed as described (29).

Construction of Transgenes and Generation of Transgenic Mice.

Details have been reported in the annual report of 1998. A schematic map of the constructs for generating transgenic mice is shown in Table 1.

Isolation of Genomic DNA , Southern Blot and PCR Analysis.

Genomic DNA was isolated from tails of 3-4 week old mice as described (22). 12 μg of genomic DNA was digested with appropriate restriction enzyme (construct A and D with Bgl II; C with Nco I and B with Acc 65I, see Table 1), electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose (Schleicher & Schuell) or Hybond N filters (Amersham). The filters were hybridized with a Sma I - Nco I fragment (734-bp) of human AP-2α cDNA which was either radioactively labeled by random-priming or labeled nonradioactively with "Genius Nonradioactive Nucleic Acid Labeling and detection Kit" (Boehringer-Mannheim). The hybridized products were visualized by autoradiography, or by light-emission, respectively. Alternatively, mice carrying the transgene were screened by PCR using primers specific for the transgene. For detection of the MMTV/AP-2α transgene, PCR was performed using forward primer MMTV-5′ (generated from MMTV LTR promoter/enhancer of plasmid pMSG, position 7591 - 7611): 5′ TCA CAA GAG CGG AAC GGA CTC 3′, and reverse primer TWY3 (generated from human AP-2α cDNA, position 111 - 118): 5′ GCT GGT GCC GTC GTC ACG 3′. PCR conditions were 1 cycle at 94°C for 1 min 20 sec; 32 cycles at 94°C for 45 sec, 53°C for 45 sec, 72°C for 2 min; and 1 cycle at 72°C for 10 min. A fragment of 158 bp was amplified. For detection of MMTV/ΔN278 and MMTV/ΔN165 transgenes, PCR was performed using forward primer MMTV-5′ and reverse primer AP2-3′ (generated from human AP-2α cDNA, position 1410 – 1437): 5′ CCT CAC TTT CTG TGC TTC TCC TCT TTG 3′. Conditions similar to the above PCR reaction were used except that the annealing temperature was 60°C. The amplified DNA fragments were 516 bp for the MMTV/ΔN278 and 821 bp for the MMTV/ΔN165.

Results and Discussion

Generation of Transgenic Mouse Lines.

As reported in my 1998 annual summary, AP-2 genes are differentially regulated during the normal development of the mouse mammary gland. We wish to further understand the role of AP-2 in the development of the mammary gland and also in tumorigenesis. Therefore we are generating mouse model systems to determine the consequences of ectopic expression of AP-2 in the mouse mammary gland. To achieve these goals we have generated four types of transgenic mouse lines, i.e., MMTV/AP-2α, MMTV/ΔN165, MMTV/ΔN278 and human c-erbB-2 promoter/rat *neu*. We wish to test the involvement of AP-2 in mammary gland development and its potential to induce mammary tumors either alone or in combination with *neu* gene using these mice.

Detection of Transgene Expression in the Transgenic Mouse Lines.

In the 1998 annual report, I showed the results of RNase protection assays on the RNA from MMTV/AP-2α, MMTV/ΔN 278 and MMTV/ΔN165 transgenic mice. Overexpression of the wild-type AP-2α was noted in the mammary glands of 6 lines. Three lines (MA44, MA7 and MA14) with higher expression level were kept for further analysis. Overexpression of the dimerization domain (ΔN278) was noted in two trangenic lines (AA2-32 and AA2-29) and overexpression of the DNA binding domain (ΔN165) was noted in one transgenic line (AA8-19). In the past year, I have optimized techniques for the detection of the protein product of the transgene by immunohistochemistry. I tested several anti-FLAG antibodies and found a rabbit polyclonal antiserum (SC-807) from Santa Cruz which worked well for this purpose. Figure 1 shows a typical result using this antiserum on mammary tissues taken from 10 day pregnant transgenic (MA44) and non-transgenic sibling mice. The expression of the transgene was clearly detected in both ductal (arrows) and alveolar (arrowheads) epithelial cells (Figure 1B). As showed in Figure 1A, the transgene product was not detected in non-transgenic mammary tissue, suggesting that the SC-807 is specific for the FLAG epitope tag.

Effects of Overexpression of Wild-type AP-2α on the Development of the Mammary Gland.

Three distinct periods of mouse mammary gland development occur postnatally (15, 24). Ductal growth occurs from 6-8 weeks of age when epithelial end buds ramify throughout the fatty mesenchyme from the nipple to create a tree-like network of ducts. The onset of pregnancy initiates the second phase of development in which lobular-alveolar structures develop from the existing ductal system. Following weaning of young, the mammary gland undergoes extensive remodeling, leading to the loss of the alveolar structures, in a process involving large scale apoptosis. In older virgin mice, 12-14 weeks of age or older, the lateral branching starts and gradually forms lobuloalveolar-like structures. However, complete development of lobuloalveolar structures occurs only during pregnancy and lactation.

In the 1998 annual summary, I reported that overexpression of the wild-type AP-2α inhibited side-branching in the virgin mouse mammary gland and also caused

defects in lobuloalveolar development. In the past year, I determined the phenotype of mammary glands taken from lactating mice.

At one day post-partum, lobuloalveoli of control mice expanded as the alveolar lumens become engorged with lactation products. In the control siblings the ducts were obscured by the extensive expansion of the lobuloalveoli (Figure 2A). Histological analysis revealed that the lumen structures formed were essentially uniform (Figure 2C). In contrast, mammary glands from the transgenic mice remained underdeveloped. Lobuloalveli were still condensed and the ducts were clearly visible by wholemount analysis (Figure 2B, arrows). Furthermore, mammary glands from the transgenic mice displayed a high degree of intragland heterogeneity (Figure 2D). A subset of alveolar lumens had a flattened appearance, indicating that the cells had undergone secretory differentiation (arrows, Figure 2D) while others were more condensed with a cuboidal appearance (arrowheads, Figure 2D). Again, fewer alveolar clusters existed in the transgenic mammary glands compared with the normal control mice (Figure 2C, D). At seven day post-partum, in normal mice, alveolar lumens occupy the whole mammary pad and stromal fat cells are rarely observed in histological sections, and the lumens are filled with secretory proteinacious material (arrows, Figure 2E). In contrast, stromal fat cells were still seen in the transgenic mammary glands (arrows, Figure 2F). Moreover, some of the alveoli from the transgenic mice at seven day post-partum still retained the cuboidal structures, and lumen products from such alveoli contained secretory lipids typically observed during late pregnancy (arrowheads, Figure 2F), indicating a delay of development. To position AP-2α in the signaling network controlling mammary gland development, I have begun a detailed histological comparison between the AP-2α transgenic mice and other mouse models. My analysis suggests that AP-2α transgenic phenotype is unique although at certain stages of mammary gland development it shares some similarity to the defects caused by overexpression of parathyroid hormone-related protein, parathyroid hormone (23) or TGF-β1 (30, 31), or to the phenotype observed in the knockout mice of inhibinβB (25) or the C/EBPβ genes (26, 27).

Overexpression of AP-2α Inhibits the Differentiation of the Mammary Epithelium.

In the mammary glands of AP-2α overexpressing mice, a subset of alveolar clusters had cuboidal epithelial cells whereas other alveoli contained more differentiated epithelia with a flattened appearance at 1 day post-partum (Figure 2D). To determine if this heterogeneity is related to AP-2α expression levels, transgene expression was determined by immunohistochemistry using the anti-FLAG SC-807 antiserum. Expression of the transgene was found in epithelial cells with a cuboidal appearance (Fig 3A, arrows). However, transgene expression was never detected in the flattened epithelial cells (Fig 3A, arrowheads), suggesting that overexpressing AP-2α inhibits the differentiation of mammary epithelium. To determine if this inhibition correlates with the expression levels of mammary differentiation markers I performed *in situ* hybridization analysis on mammary tissue taken from 1 day lactating mice. My analysis showed that the expression of the whey acidic protein gene and the β-casein gene were heterogeneous. Some clusters of alveoli had significantly lower expression

levels and these clusters seemed to be less differentiated (Figure 4A, B; arrows). However, it is necessary to determine if the expression of the transgene co-localizes with these clusters of alveoli and these experiments are underway.

Despite the dramatic inhibition of side-branching of virgin mammary glands, and lobuloalveolar development during pregnancy and lactation in the mice overexpressing AP-2α, there was no apparent consequence for the trangenic mothers to feed full-size litters. The explanation for this phenomenon may be the incomplete inhibition of terminal differentiation observed at one day post-partum caused by variegated transgene expression, and the ability of the trangenic mammary glands to catch-up in late lactation.

Effects of Overexpression of a Dominant-negative Form (ΔN278) of AP-2 on the Development of the Mammary Gland.

I obtained two transgenic mouse lines (AA2-32 and AA2-29) which overexpressed the dimerization domain of AP-2α in the mammary gland. I analyzed the mammary gland phenotype of these transgenic mice at virgin and pregnant stages. As shown in Figure 5, overexpression of ΔN278 also impaired the formation of lateral branches in virgin mice. In contrast to the inhibition caused by the wild-type AP-2α, the inhibitory effects of ΔN278 did not extend to the pregnant stage. At 14 days of pregnancy, no significant phenotype differences could be determined between the mammary glands taken from transgenic (Figure 5D) and non-transgenic sibling (Figure 5C) mice. These data are still preliminary and we need to confirm appropriate transgene expression using the anti-FLAG antiserum. It has been shown that the dimerization domain of AP-2α can dimerize with all three proteins in the AP-2 family (19). Therefore, the ΔN278 product in the mammary gland could dimerize with AP-2α, AP-2β and AP-2γ proteins and inhibit their function. Though we showed above that overexpression of AP-2α resulted in inhibitory effects on the mammary gland development and morphogenesis, AP-2γ or AP-2β could play a stimulatory role during the development of the mammary gland by activating different downstream targets. There is evidence that AP-2 family members can preferentially activate different targets *in vivo* (28). Therefore, inhibition of AP-2γ or AP-2β could cause inhibitory effects on the development of the mammary gland. As reported last year, the expression of endogenous AP-2 genes increases significantly during pregnancy. Therefore, an alternative explanation is that there may not be sufficient ΔN278 product to neutralize the activities of endogenous AP-2 proteins during this developmental stage. In future experiments, it may be worthwhile to overexpress other AP-2 proteins, especially AP-2γ, in the mammary gland to test the hypothesis that AP-2γ plays a stimulatory role during the development of the mammary gland.

Effects of Human c-erbB-2 Promoter/Rat *neu* Transgene on the Development of the Mammary Gland.

In my 1998 annual summary, I reported a constant phenotype of overgrowth of the mammary ductal network in virgin mammary gland from transgenic mice carrying

the human c-erbB-2 promoter/rat *neu* transgene. By crossing these transgenic mice with the mice overexpressing AP-2α, doubly transgenic mice were obtained. The overexpressed AP-2α appeared to inhibit overgrowth caused by neu gene. However, these phenotypes were not observed in the mammary glands taken from pregnant mice (not shown). I have not been able to detect the expression of *neu* in AP-2α singly and AP-2α/*neu* doubly transgenic mice. One explanation for this finding is that *neu* levels are usually very low in the normal mammary gland. Alternatively, AP-2α alone may not be sufficient to cause the overexpression of *neu*. Instead, it may be necessary to overexpress both AP-2α and AP-2γ in the mammary gland to transactivate *neu* gene. Our data from the analysis of human breast cancer samples support such a hypothesis, as there is a significant correlation between the overexpression of c-erbB-2 and the presence of both AP-2α and AP-2γ (28). Finally, no tumors have been found in either *neu* singly transgenic or *neu*/AP-2α doubly transgenic mice followed for more than one and a half years, suggesting that the expression levels of *neu* gene in these transgenic mice are low.

In conclusion, my research strongly supports a role for AP-2α in the normal development of the mammary gland. In order to understand the molecular mechanisms resulting in impaired development of the mammary gland it will be necessary to determine potential downstream targets for AP-2α transcriptional control. To achieve this goal, I have collected a number of antibodies and RNA probes and I am in a process of determining such potential targets. The AP-2 proteins are a class of transcription factors that are thought to control genes involved in cell:cell contact, cell:cell signaling, cell proliferation and differentiation. The MMTV/AP-2α mouse model provides a valuable tool to study the role of these cellular processes in the proliferation, differentiation and morphogenesis of the mammary gland. It is also of particular interest to determine if the presence and expression of the MMTV/AP-2α transgene could suppress tumor formation by crossbreeding these mice with MMTV/oncogene or WAP/oncogene mice.

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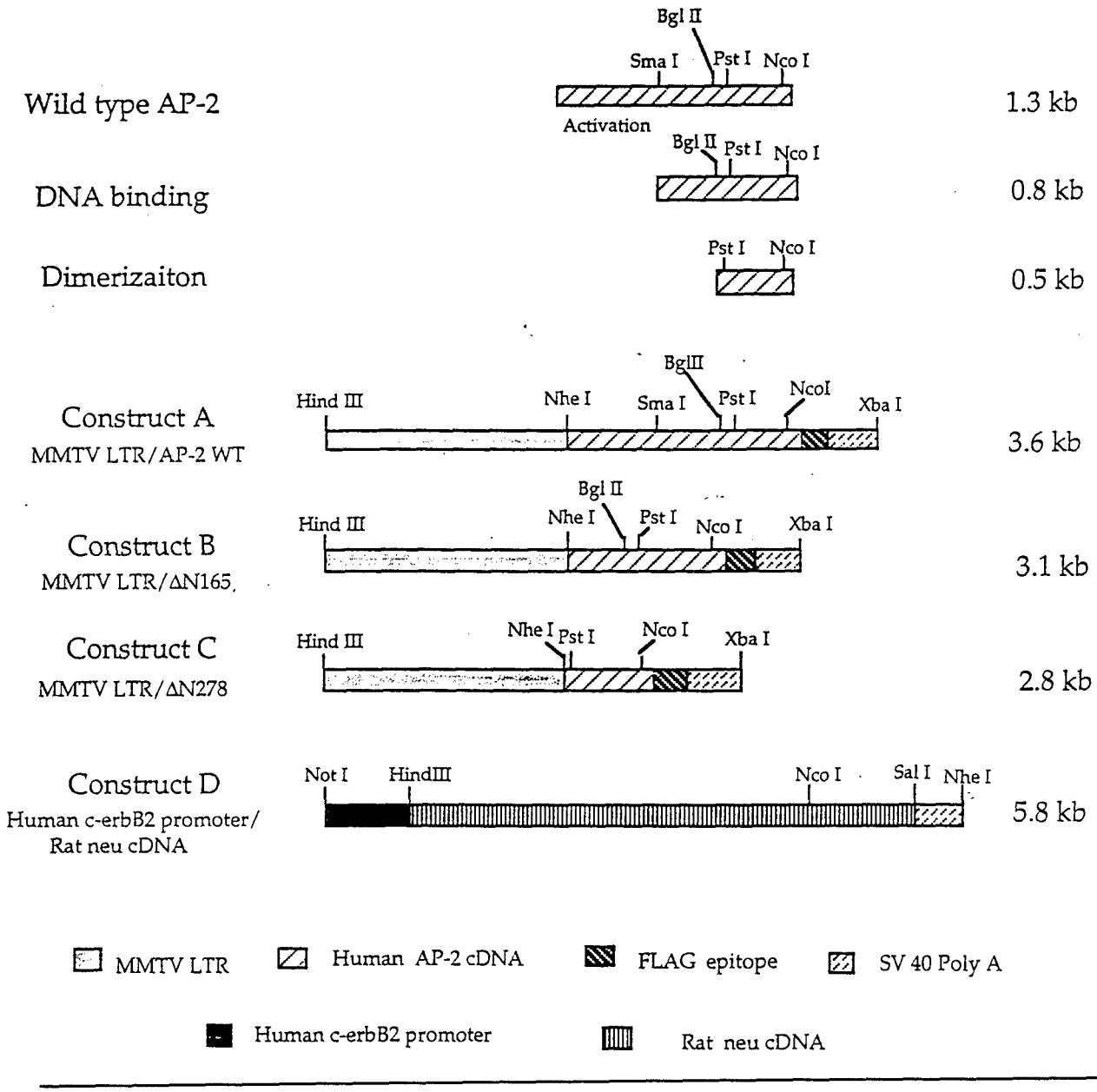
Table 1. Constructs for generating transgenic mice.



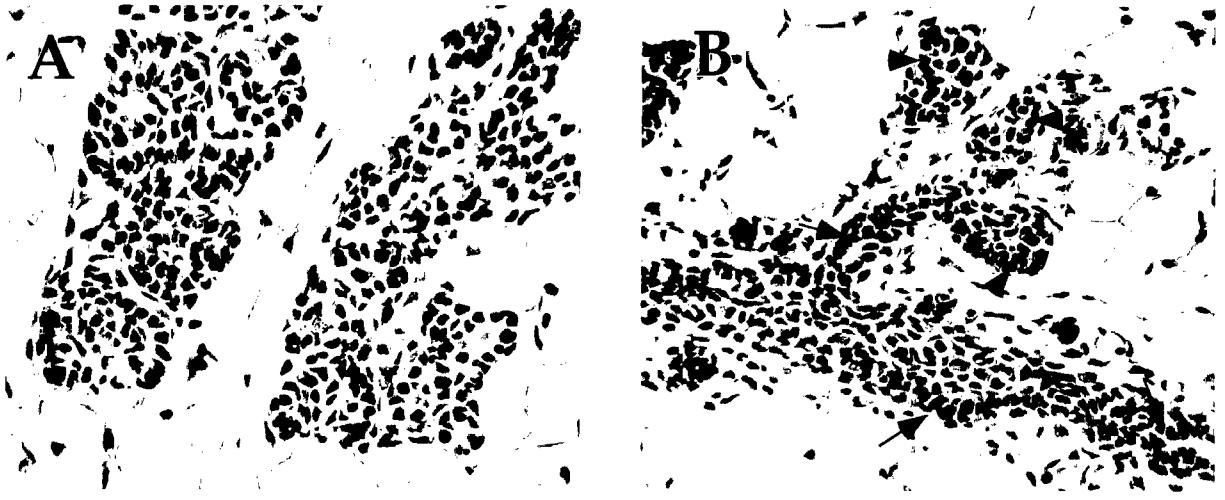


Figure 1. Expression of MMTV/AP-2α transgene. Immunohistochemistry using the SC-807 on 10-day pregnant mammary tissues from non-transgenic sibling (A) and transgenic (B) mice. Note the expression of the transgene in the mammary epithelium of the transgenic mice (brown staining) but not in the mammary tissue of the normal siblings (A).

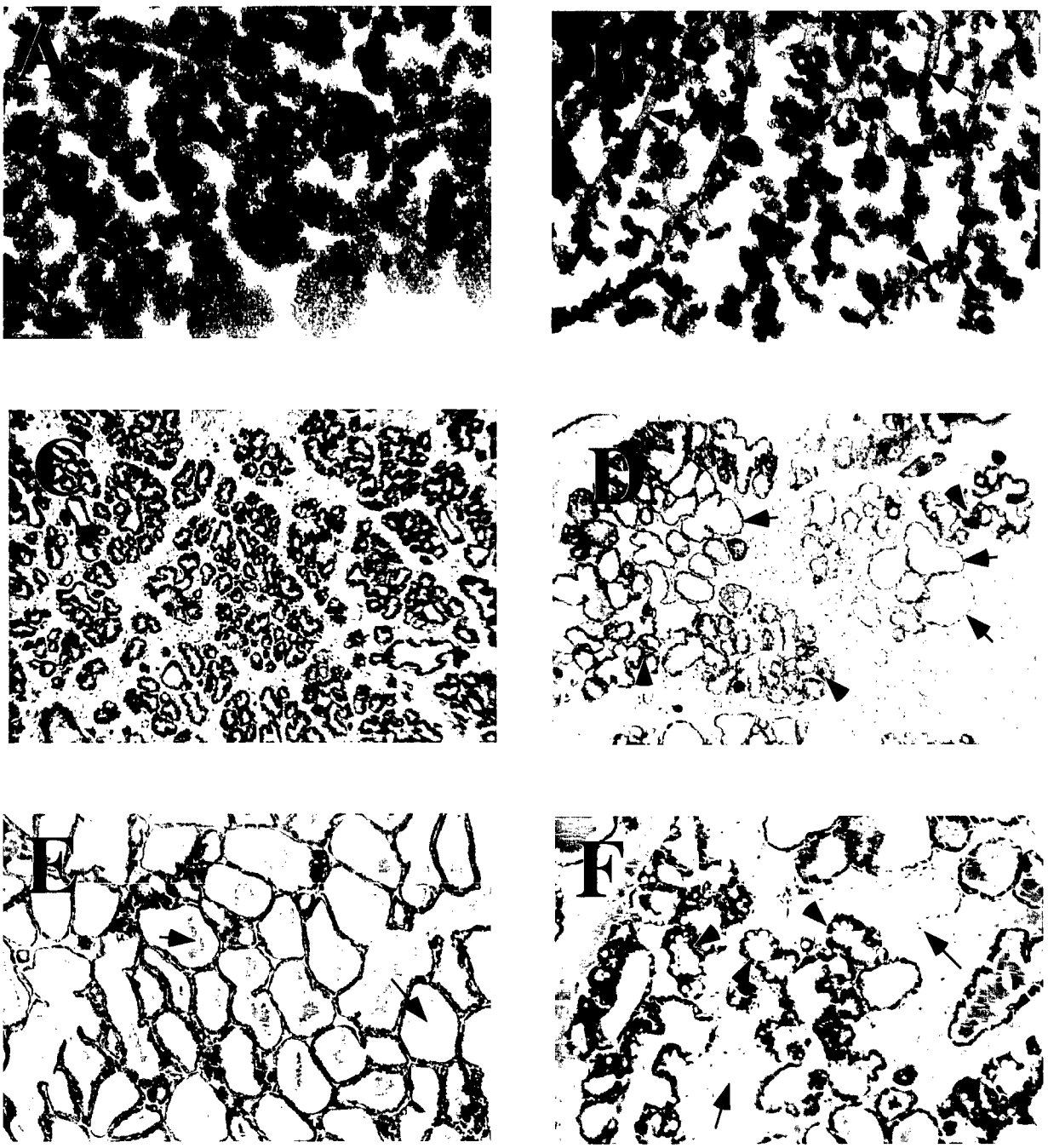


Figure 2. Analysis of the 4th inguinal mammary glands taken from normal sibling (A, C, E) and transgenic (B, D, F) mice. (A, B) Wholemount analysis of the mammary glands at 1 day post-partum. Arrows in (B) indicate the visible ducts. (C, D) Section of (A, B). Arrows in (D) indicate more differentiated alveolar lumens while arrowheads indicate less differentiated alveolar clusters. (E, F) Section of the mammary tissues taken from 7 day post-partum mice. Arrows in (E) indicate the secretory proteinacious materials in the normal lumens. Arrows in (F) indicate the stromal fat cells while arrowheads indicate the under-developed lumens containing secretory lipids in the transgenic mammary glands.

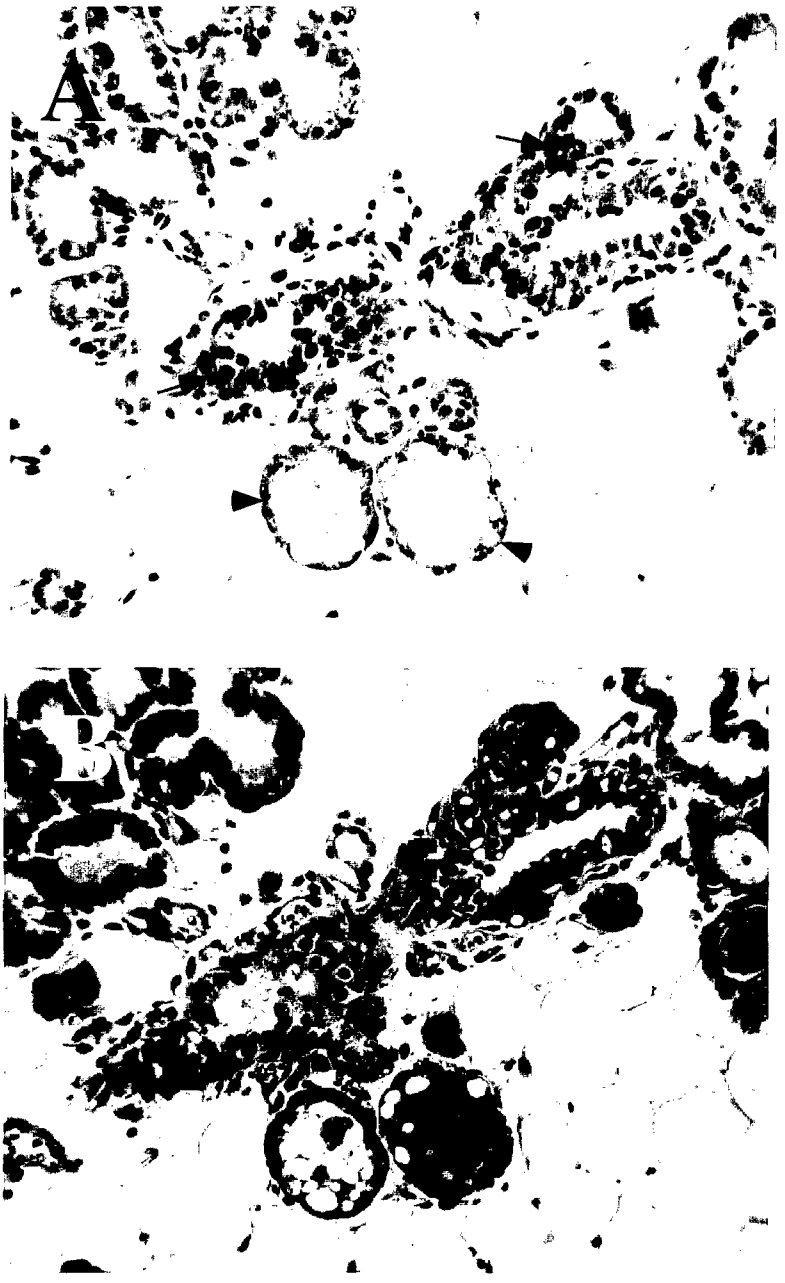


Figure 3. Overexpression of AP-2α inhibits the differentiation of the mammary epithelium. 1 day post-partum mammary tissue of transgenic mouse was sectioned and stained with anti-FLAG antiserum SC-807 (A). (B) Adjacent section of (A) stained with H + E. Note the expression of the transgene in the premature mammary epithelium (arrows) but not in more differentiated mature mammary epithelium (arrowheads).

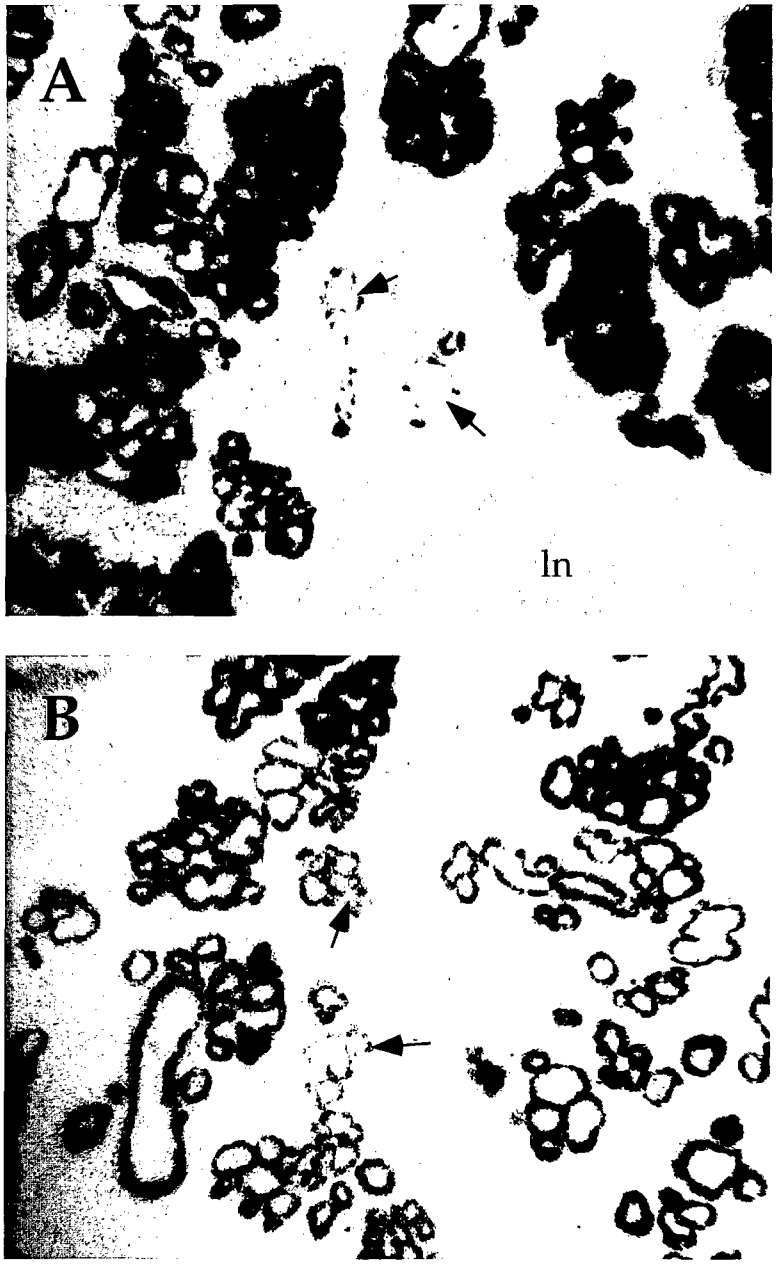


Figure 4. In situ hybridization analyses of whey acidic protein gene (A) and β-casein gene (B) expression in the mammary glands taken from 1 day lactating transgenic mice. Arrows indicate the clusters of alveoli with lower expression levels of the differentiation markers. ln: lymph node.

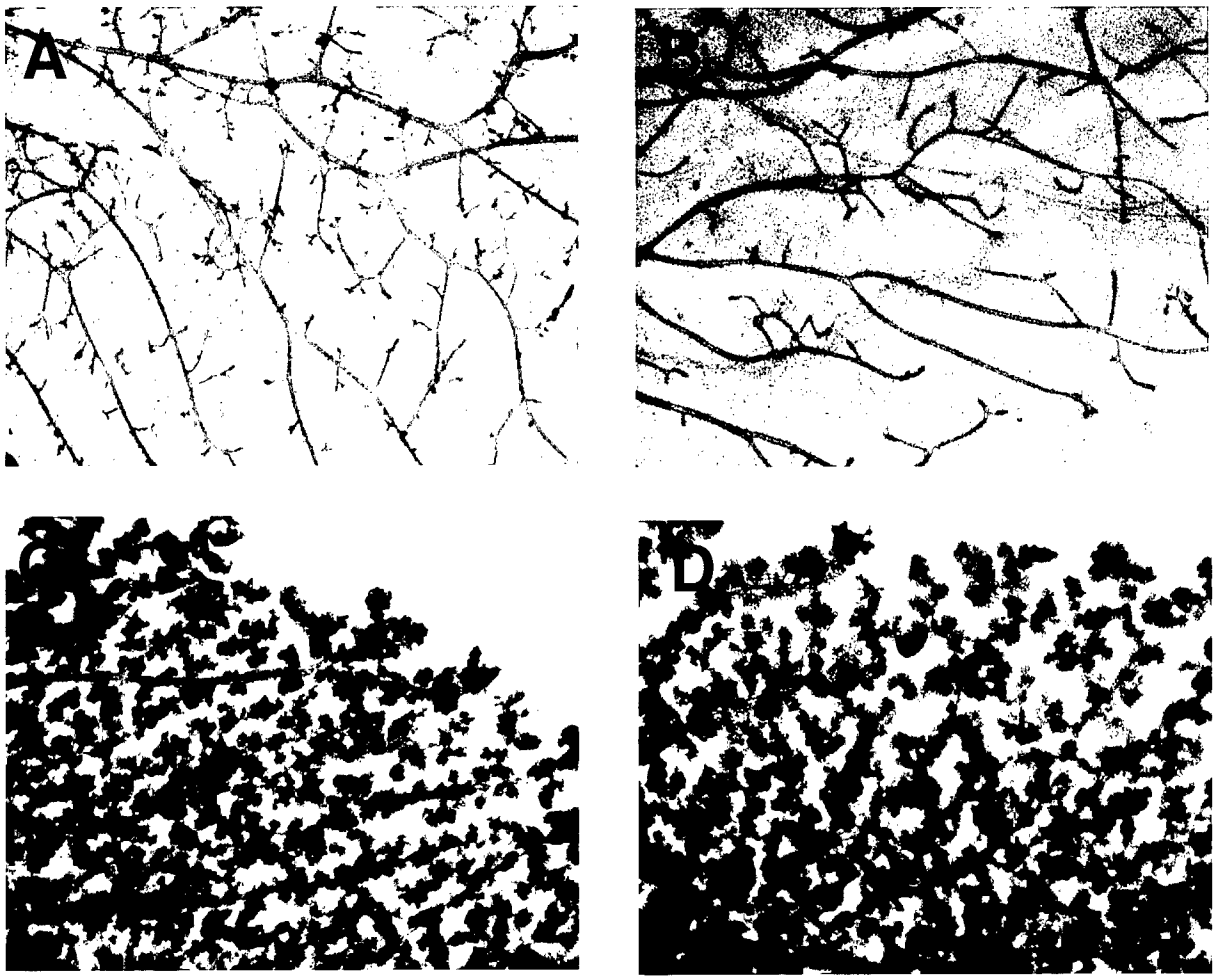


Figure 5. Whole-mount analysis of the forth inguinal mammary gland taken from normal littermate (A, C) or trangenic (B, D) virgin mice. (A, B) Mammary tissues taken from 5 month old virgin mice. (C, D) Mammary tissues taken from 14 day pregnant mice. Note that the transgenic virgin ducts have fewer side branches (B) as compared with the non-transgenic gland (A). There is no significant morphological differences between the transgenic and non-transgenic glands at 14 day pregnancy.